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(54) **Title:** CLINICAL ASSAY FOR NUCLEIC ACIDS AMPLIFIED IN SOLID MATRICES TO PRODUCE COLONIES OF THE PROGENY OF INDIVIDUAL TARGET MOLECULES

(57) **Abstract:** A quantitative multiplex assay for at least two nucleic acid targets, on of which is rare and another of which is present at much higher level, comprising adding reagents for amplification of the targets by polymerase chain reaction (PCR) to a thin polyacrylamide gel matrix that has been washed and dried, distributing a sample containing the targets across the surface of the gel sufficiently to separate rare target molecules but not molecules of the abundant target, cycling the gel through a PCR protocol to amplify the target, and counting the number of individual colonies of the rare target.

CLINICAL ASSAY FOR NUCLEIC ACIDS AMPLIFIED IN SOLID MATRICES TO PRODUCE COLONIES OF THE PROGENY OF INDIVIDUAL TARGET MOLECULES

FIELD OF THE INVENTION

This invention relates to diagnostic assays for nucleic acids that include amplification by the polymerase chain reaction (PCR).

BACKGROUND

The method for amplifying nucleic acids in solid matrices to produce colonies of the progeny of individual target molecules is disclosed in our United States Patent No. 5,616,478 for Method for Amplification of Nucleic Acids in Solid Media, which is incorporated by reference herein in its entirety. That patent discloses various techniques for spreading out nucleic acids in a solid matrix, adding an amplification system to the matrix, and amplifying to produce colonies of amplified target. Among the solid matrices disclosed are various packed beds of granules, lamellae or filaments, and thin layers. The solid matrices may be of a variety of materials such as agarose, polyacrylaminde, nylon, gelatin, alginate, carrageenan, cellulose, silica gel, titanium sponge, cross-linked agarose, dextran or polyethylene glycol. The amplification system may be any system for exponential amplification of nucleic acids in vitro, such as viral RNA-directed RNA polymerases, PCR or isothermal multienzyme (3SR) amplification, and may be introduced into a gel matrix either prior to gel formation or, particularly when the conditions of gel preparation are too strong for labile amplification reagents, by impregnation of pre-cast gels.

Of the various amplification methods disclosed in United States Patent No. 5,616,478, the best known and widely used is PCR, which finds wide use in a variety of solution amplification methods (S-PCR).

SUMMARY OF THE INVENTION

An aspect of this invention is a type of multiplex clinical assay for relatively rare nucleic acid targets residing in samples (for example, samples of blood from a human patient) containing other target nucleic acids and non-target nucleic acids, which are present in manifold excess compared to the relatively rare target. The

diagnostic assay utilizes the polymerase chain reaction (PCR) process. If the target nucleic acid is RNA, reverse transcriptase-PCR (RT-PCR) is used, of course.

In our clinical assay method, nucleic acids (DNA, RNA) isolated from a sample need to be diluted in the solid matrix, by spreading, only sufficiently to permit the growth of individual colonies from the relatively rare target molecules. Nucleic acids from the sample need not be diluted sufficiently that other, more abundant nucleic acid targets would form identifiable individual colonies in a multiplex assay of both targets (or all targets, if more than two are amplified). For example, if a sample contains nucleic acids from two viruses, one can amplify nucleic acid target from the rarer of the two to form identifiable individual colonies even though the more abundant of the two is present in such a large concentration, ten or more times as much, that even without amplification it appears on probing to permeate the matrix entirely. Multiple amplifications can take place simultaneously in the matrix without affecting the number of colonies from each target, only the colony size. The same number of colonies of a rarer target nucleic acid arise even when colonies from a more abundant target merge into an undifferentiable mass. Further, unintended (false) priming of human genomic DNA in the sample, even in huge excess, does not interfere with the assay, which relies on counting the number of colonies produced from rare target nucleic acid.

In the assay of this invention each target is amplified by a pair of primers that, false priming aside, is specific to that target relative to other nucleic acids in the sample. Thus, in one embodiment two different viral targets, one rare and one abundant, are amplified using a pair of primers specific to each, and colonies of each are identified utilizing probes specific to each. In another embodiment, a rare mutant allele can be detected in a sample containing abundant wild-type allele by using a primer pair that is specific for the mutant relative to the wild type. For mutants differing from their corresponding wild types by a single nucleotide, one could utilize a primer whose 3' terminal nucleotide is complementary to the mutant at that single nucleotide. Alternatively, one could utilize a high specificity primer such as is disclosed in published PCT patent application WO 00/71562 A1.

For quantitative results, one can amplify the DNA in a sample utilizing a dilution series to reach the point where individual colonies of the rare target can be

counted. Alternatively, one could determine the total amount of DNA that is present in a sample spectrophotometrically. For the abundant target further dilution of the sample, as by a dilution series as indicated, can be made until it also produces individual colonies, which can then be counted for quantitative results. Additional targets, if present, having concentrations intermediate the rare target and the abundant will produce countable quantities at intermediate dilutions, such as occur with a dilution series.

The multiplex assays of this invention utilize polyacrylamide gel in a thin layer as the solid matrix. Further, the polyacrylamide gel is pre-cast, washed and dried as a plain gel, and later impregnated with PCR reagents prior to use, which we have found to impart improved reproducibility, important for diagnostic assays, as compared to mixing the amplification reagents with gel-producing ingredients prior to casting.

Preferred embodiments of assays according to this invention also utilize improved nucleic-acid isolation from the sample, if a blood sample, that includes washing pellets of isolated nucleic acids with a saline-alcohol solution, which we have discovered removes substances that are inhibitory to PCR and reverse transcription.

DETAILED DESCRIPTION

In clinical practice, there is a need for a reliable, sensitive, and quantitative assay for targets varying widely in concentration (or copy number), including pathogenic viruses. Ideally, the assay would tell a doctor if a patient is infected, with which viruses, and how many copies of each virus are present in the patient's body at a moment. Such an assay is reported here. It comprises not just an improvement of earlier methods, but a *new* approach based on the molecular colony technique (MCT) invented in this laboratory[1] (superscripts identify references, listed below). According to the developed protocol, nucleic acids extracted from a specimen are introduced into a thin polyacrylamide gel, together with, for example, *Thermus thermophilus* (Tth)DNA polymerase (which can function both as a DNA polymerase and a reverse transcriptase in the presence of Mn^{2+} ions)[2,3], 2'-deoxyribonucleoside 5'-triphosphate (dNTP) substrates, and virus-specific oligonucleotide primers. The gel is incubated under conditions appropriate for reverse transcription (RT), followed by polymerase

chain reaction (PCR)[4] temperature cycles. During incubation, target molecules (in this embodiment, viral DNA or RNA)produce DNA colonies at discrete locations within the gel. The identity of DNA colonies is revealed by hybridization with virus-specific labeled probes. The number of DNA colonies hybridizable with a particular probe indicates the copy number of the respective viral DNA or RNA in the analyzed sample. Compared to the conventional solution PCR (S-PCR), the new method shows better sensitivity and reliability, eliminates the interference between assayed targets and from the background DNA synthesis caused by primer-dimers or by mispriming on patient's own nucleic acids, and provides for a direct determination of target titer.

MCT is a method of amplifying nucleic acids (RNA or DNA)in a matrix, which may be a gel. In this format, the progeny of each molecule forms a colony, rather than spreads throughout the reaction volume. Each colony comprises many copies of one original molecule (i. e., a clone), and the number of colonies indicates the number of nucleic acid molecules initially present in the gel. The unique feature of MCT distinguishing it from other methods for nucleic acid amplification is that amplified molecules are spatially separated. This results in weakening or (given the sample is properly diluted) complete elimination of the competition between molecular species, and allows individual amplifiable molecules to be monitored, counted and analyzed.

In the first published version of MCT (refs. 5,6), the gel was agarose containing Qβ replicase (the RNA-directed RNA polymerase of bacteriophage Qβ)[7] and ribonucleoside 5'-triphosphates. Qβ-MCT provides for growing the colonies of RQ RNAs (i. e., RNAs Replicable by Qβ replicase, including the natural Qβ phage satellites[7]), and proved to be a powerful tool for the *in vitro* studies of very rare events of RNA recombination[8,9]. In principle, it is possible to use Qβ-MCT for diagnostic purposes, e. g., by employing the protocol in which an RQ RNA carrying a target-specific insert is generated by ligating its nonreplicable fragments that have been hybridized next to each other on a target RNA or DNA[10]. However, such an approach is of a limited use because it is technically complicated, the RNA fragments are intrinsically recombinogenic and can produce RQ RNAs in the absence of a target and ligase[8,9], and many foreign inserts, even short, do inhibit replication of RQ RNAs due to the structural requirements that Qβ replicase imposes on its templates[7,11].

These drawbacks are overcome in PCR-MCT according to the present

invention, in which DNA colonies from two or more targets are grown by carrying out PCR in heat-resistant polyacrylamide gel. In contrast to the RNA amplification by Qβ replicase, virtually any DNA can be amplified in PCR, which is now widely used in basic research, biotechnology and clinical diagnostics[12]. Recently, our original PCR-MCT protocol[1] has been reproduced by others[13] and, inasmuch as the equipment and reagents that are required for carrying out PCR are readily available on the market, we believe that PCR-MCT can become a routine laboratory technique.

Efficiency of DNA and RNA detection

We checked the performance of PCR-MCT by assaying human hepatitis B virus (HBV) DNA, human immunodeficiency virus (HIV-l)RNA and Qβ RNA; the first two were cloned fragments of conservative viral sequences and the latter was the entire genome of Qβ phage. The number of target-specific DNA colonies was close to the number of DNA molecules added to the gel (101 ± 14% for HBV DNA, *n* =4); the recovery of RNA molecules was lower and similar for the two RNAs tested (13 ± 3% for HIV-1, *n* =13; and 15 ± 3% for Qβ, n =4), see Fig. 1. Since we have increased RNA recovery to that high level as a result of the attempts to avoid ribonuclease contaminations (see Examples), we believe that it can be further increased by additionally purifying the protein preparations used in the assay, Tth DNA polymerase and bovine serum albumin (BSA). By comparing the signals produced by DNA colonies with those by known amounts of S-PCR products spotted on the blotting membrane (not shown) we have determined that each colony contains 10^7 to 10^8 replicas of the progenitor template.

Mixed targets

In practice, it is useful to simultaneously assay (a multiplex assay) several viruses or other targets whose load may differ by one or more orders of magnitude. Therefore, we checked if the presence in the gel of more than one targets, with their respective primers, would result in their mutual competition for polymerase and substrates. Surprisingly, we observed no interference to the amplification of one species (HIV-l RNA) from the comcomitant amplification of another (HBV DNA), even if the latter was greater than a million-fold more abundant (Fig. 2). Fig. 2 shows that 300 molecules of rare target lead to countable colonies, whereas 1000

molecules of the abundant target have begun to produce merged colonies. However, in a multiplex assay in which both targets are amplified, the rare target still produces, reliably and reproducibly, countable colonies when the colonies of the abundant target have totally blurred. This result demonstrates a very high intrinsic resolving power of the PCR-MCT gel: even though the visible diameter of a DNA colony on the autoradiogram is 0.1 to 1 mm, no competition is observed at a density of 16 x 10^6 colonies per mm^3 (10^9 HBV DNA molecules in the 62 μm gel volume, the largest amount tested), indicating an effective colony diameter that is under 4 μm.

Tolerance to human nucleic acids

In real assays, a few viral or other target molecules are to be detected in samples containing huge amounts of human (or other irrelevant)DNA and RNA. Given the complexity of the human genome and a limited specificity of hybridization between nucleic acids, it is not surprising that target-specific (e.g., virus-specific) oligonucleotides anneal (despite some mismatches)and prime on human sequences resulting in a background DNA amplification. The background amplification can be monitored by gel electrophoresis of the reaction products, and it interferes with the amplification of a target in S-PCR assays (Fig. 3a). In contradistinction, no interference is seen in the multiplex MCT format according to this invention, even when the amount of human nucleic acids is 100 billion times larger than that of the rare target, see Fig. 3b. Of course, in this case the background amplification does occur, but is mostly restricted to gel areas not overlapping with those occupied by target DNA colonies.

Although PCR technology is already a highly developed field, it would greatly benefit from the features provided by multiplex MCT according to this invention. The multiplex MCT method according to this invention is able to overcome the conceptual problems inherent to S-PCR, such as the sensitivity loss due to a competition from background amplification (caused by false priming on non-target sequences often present in the sample in a great excess over the target), preferential amplification of some templates over others, and template recombination (during the amplification of heterozygous samples and multigene families, and in multiplex PCR).[14] In MCT, different molecules of the same target amplify at different

locations, which eliminates any competition and prevents recombination between them. In multiplex assays according to this invention, we have discovered that this holds true even when another target is present in high amount and not spread sufficiently to produce identifiable colonies. The advantages of the MCT format are even more obvious in quantitative assays. (i)MCT directly determines the number of target molecules, whereas S-PCR measures it indirectly, by referring to the signal produced by a known number of molecules of an internal standard (IS) added to the sample; therefore, the result depends on whether the target and IS are equally efficient templates. In addition, due to the mutual template interference, reliable measurements can only be done if the quantities of the two templates differ by ≤1.5-fold; therefore, careful multiple-reaction calibrations need to be carry out to assess a single target species in one sample.[15] (ii) This problem exaggerates in the multiplex S-PCR assaying for two or more targets that may differ both in the amplification rate and in the copy number; in contrast, provided that the sample is properly diluted, MCT eliminates any interference between individual target molecules (as these are physically separated)and differing amplification rates will only affect the size of DNA colonies. (iii)While MCT counts the number of colonies, S-PCR relies on the signal intensity measurements which are inherently error prone. (iv)In contrast to the linear relationship between the number of templates and the number of colonies in MCT, the signal intensity in S-PCR is a logarithmic function of the initial template number; accordingly, errors in the target determinations are much greater than those in signal measurements. (v) S-PCR becomes even less reliable when the number of target molecules is low, because of the stochastic nature of every PCR step (primer annealing, its elongation, and melting DNA duplexes); as a result, the target can be either under-or over-amplified compared to IS. In MCT, the stochastic effects can only result in colony size variations. Surprisingly, these advantages remain in a multiplex assay even when only the rare target is sufficiently diluted to produce countable colonies.

EXAMPLES

The following description applies to assays reported in Figs. 1-3 and discussed above.

DNA and RNA targets. As an HBV DNA target, we used plasmid obtained by

ligating, into pTZ19R BarnHI-fragment encoding core antigen HbcAg, the HBV subtype ayw genome[16] that had been excised from pUHBc (ref. 17). As an HIV-I RNA target, we used a 879 nt-long run-off transcript from a *Sma*I-digested plasmid[10] carrying, downstream from T7 promoter, fragment 4230-5091 of HIV-I strain NL4-3; the transcript has been treated with RQ1 Rnase-free DNase (Promega, Madison, WI) to eliminate the plasmid DNA; the resulting preparation produced no specific PCR product if RT step was omitted. Qβ RNA was isolated by phenol extraction from wild-type Qβ phage that was purified as described[5].

Oligonucleotides. For target amplification: 5'-GCTTAATACGACTCACTATAGGGCTA- CTGTGGAGTTACTCTCGTTTTTGC-3' and 5'-GTCTATAAGCTGGAGGAGTGCGA- ATC-3' (matching positions 1933-l 960 and 2277-2302 of the HBV genome, respectively); 5'-CAAGTAGACTGTAGTCCAGGAATAT-3' and 5'-GCTTAATACGACTCACTATAG- GGGATTGTAGGGAATGCCA-3' (matching positions 4386-4410 and 4646-4665 of the HIV genome, respectively); 5'-GCCGTCATCGTCATGGCATATCTT-3' and 5'-GCTTAATACGACTCACTATAGGCCGCCCATTTCGTTCAC-3' (matching positions 869-892 and 1145 - 1164 of Qβ RNA, respectively); the longer primers introduced T7 promoter (underlined)that provided for *in situ* transcription of DNA colonies. For hybridization with DNA colonies: 5'-CAATGCTCAGGAGACTCTAAGGCTTCCCGATACAGAGCTG-3' (positions 2006-2045 of HBV genome) and 5'-CAGTACATACAGACAATGGC-AGCAATTTCACCAG-3' (positions 4564-4597 of HIV genome).

DNA polymerases. The coding region of *polA* gene (GenBank accession No. D28878) was PCR-amplified, starting with chromosomal *T. thermophilus* HB8 DNA, using primers that introduced upstream site NdeI and His_6-coding sequence and downstream site EcoRV, cloned in pET11c between sites NdeI and *Bam*HI and expressed in B834(DE3)/pLysS *Escherichia coli* cells[18]. Highly purified N-His_6-tagged Tth DNA polymerase was isolated from these cells by heating lysate and $(NH_4)_2SO_4$ precipitation[19], followed by chromatography on Zn^{2+}-iminodiacetate-Sepharose[20]. N-His_6-tagged Taq DNA polymerase was obtained similarly.

Isolation of nucleic acids from total blood. We used anonymous citrate-treated blood samples obtained from healthy donors and tested HBV and HIV-1 negative at a blood transfusion station; IRB required no informed consent in this case. We added a blood aliquot into 4 volumes of a hot lysis solution (125 mM Tris-HCl pH 7.5, 125 mM NaCl, 12.5 mM EDTA, 6.25% SDS, 2.5%2-mercaptoethanol), incubated for 3 min in a boiling bath; then for 1 h at 65 °C in the presence of 0.2 mg/ml proteinase K (Amersham-Pharmacia Biotech, Moscow, Russia)and again for 3 min in a boiling bath, extracted the sample (after adjusting NaCl to 300 mM) with an equal volume of phenol: chloroform: isoamyl alcohol mixture (24:24:1 v/v/v) and twice with an equal volume of chloroform; precipitated with 3 volumes of ethanol (for blood samples <1 ml, linear polyacrylamide[21] was added to 0.1 mg/ml prior to ethanol for easier pellet visualization); twice extracted the pellet (10 min at room temperature, with occasional vortexing) with 0.5 volumes (of the original blood aliquot)of a mixture of ethanol:(200 mM Na-citrate, 300mM NaCl) (45:55 v/v; this discolored the pellet and, we discovered, removed substances inhibitory to PCR); washed the residue with 80% ethanol, suspended it in 0.1 mM Na-EDTA (pH 8.0) and heated for 2 min in a boiling bath. Residual ribonucleases were inactivated with iodoacetamide[22] at pH 8.0. The yield of total nucleic acids (DNA +RNA)was 30-60μg per ml of blood, as determined spectrophotometrically. We tested the procedure by introducing a plasmid DNA and Qβ phage prior to proteinase K treatment, and observed no appreciable loss or degradation of the exogenous nucleic acids.

DNA colony growth and detection. We cast polyacrylamide amplification gels (8% acrylamide/0.12% N,N'-methylene-*bis*-acrylamide) in shallow wells (0.4 mm deep, 14 mm diameter)made in a 1.5 mm thick microscopic glass slide and treated with PlusOne Bind-Silane (Amersham-Pharmacia Biotech), washed them in deionized water, autoclaved and dried *in vacuo* at room temperature. Prior to amplification, we reconstituted the gels by swelling (during 1.5 h at 4 °C, under cover glasses for microscopy, sealed with a silicone grease)in 65 μ1 of RT-PCR cocktail [40 mM Bicine-KOH (pH 8.3), 90 mM K-acetate, 7%glycerol, 1 mg/ml bovine serum albumin "for molecular biology " (Roche Molecular Biochemicals, Mannheim,

Germany), 2.5 mM MnC12, 0.28 mM each of dCTP, dGTP and dTTP, 0.56 mM dATP, 0.45 μM primers, 0.4 μg Tth DNA polymerase, 0.08 μg Taq DNA polymerase, and a template], placed the slides into UN0 *in situ* Thermocycler (Biometra, Göttingen, Germany), on a flatbed thermoblock preheated to RT temperature (56 °C for HIV-l RNA or 64 °C for Qβ RNA, either for HBV DNA). After 30 min incubation, PCR cycles were performed as follows: the first 3 cycles [melting (94 °C, 15 s), annealing (4 s) and extension (72 °C, 60 s)] were followed by 40 cycles with the melting time being reduced to 6 s, followed by incubation at 72 °C for 5 min. Annealing temperature was 50 °C for HIV-l target or 60 °C for Qβ target, either for HBV target. After PCR, we blotted the gels (20 min) with Hybond N+ nylon membrane (Amersham-Pharmacia Biotech), fixed the membrane in 80% ethanol (2 min), dried, placed them for 3 min on a filter paper moistened with denaturing solution (0.5 N NaOH, 10 mM EDTA), then on a filer paper with 2x SSC (ref. 23), dried again, and UV-crosslinked and hybridized as described[6]. For consecutive hybridizations, a previous probe was stripped away by washing the membrane in denaturing solution (20 min), and then in 2x SSC (3 min). RNA hybridization probes were prepared by T7 RNA polymerase transcription in the presence of [α-^{32}P]ATP (ref. 6) using the above plasmids carrying HBV and HIV-1 sequences, and plasmid pQβ7 carrying Qβ cDNA downstream from T7 promoter[24]; oligonucleotides were labeled using [γ-^{32}P]ATPand polynucleotide kinase (Roche Molecular Biochemicals)according to the manufacturer 's protocol.

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What is claimed is:

1. A quantitative multiplex polymerase chain reaction (PCR) diagnostic assay for at least two target nucleic acid sequences, a rare target and an abundant target, that differ in abundance by at least an order of magnitude, comprising

a) preparing an isolate of nucleic acids from a sample;

b) providing a first thin-layer polyacrylamide gel that has been cast, washed, dried, and impregnated with a PCR amplification mixture that includes at least polymerase enzyme, deoxyribonucleotide triphosphate substrates, and a primer pair specific to the rare target, and a primer pair specific to the abundant target;

c) distributing said isolate across the surface of said first gel sufficiently to permit growth of separate, detectable colonies of amplified rare target but insufficient to permit growth of separate, detectable colonies of amplified abundant target;

d) thermally cycling said first gel through sufficient PCR cycles to produce detectable colonies of said rare target; and

e) counting the number of colonies of said rare target.

2. The method of claim 1, wherein said step of counting colonies includes hybridizing target-specific labeled probes to colonies of said rare target.

3. The method of claim 1, or claim 2 wherein said rare target is RNA and wherein said PCR cycles are preceded by reverse transcription of said RNA.

4. The method of any of claim 1-3 further comprising

f) providing a second thin-layer gel according to step b);

g) distributing said isolate across the surface of said second gel sufficiently to permit growth of separate, detectable colonies of amplified abundant target;

h) thermally cycling said second gel through sufficient PCR cycles to produce detectable colonies of said abundant target; and

i) counting the number of colonies of said abundant target.

5. The method of claim 4 wherein steps c) and g) are performed by distributing a dilution series of said isolate across a series of said thin-layer gels.

6. The method of claim 4 wherein said steps e) and i) include hybridizing target-specific labeled probes to colonies of said rare target and said abundant target, respectively.

7. The method of any of claims 4-6 wherein said abundant target is RNA and wherein said PCR cycles are preceded by reverse transcription of said RNA

8. The method of any of claims 1-7 wherein said sample is a whole blood, and preparing said nucleic acid isolate includes precipitation of nucleic acids with alcohol to form a pellet and washing the nucleic acid pellet with a saline-alcohol solution.

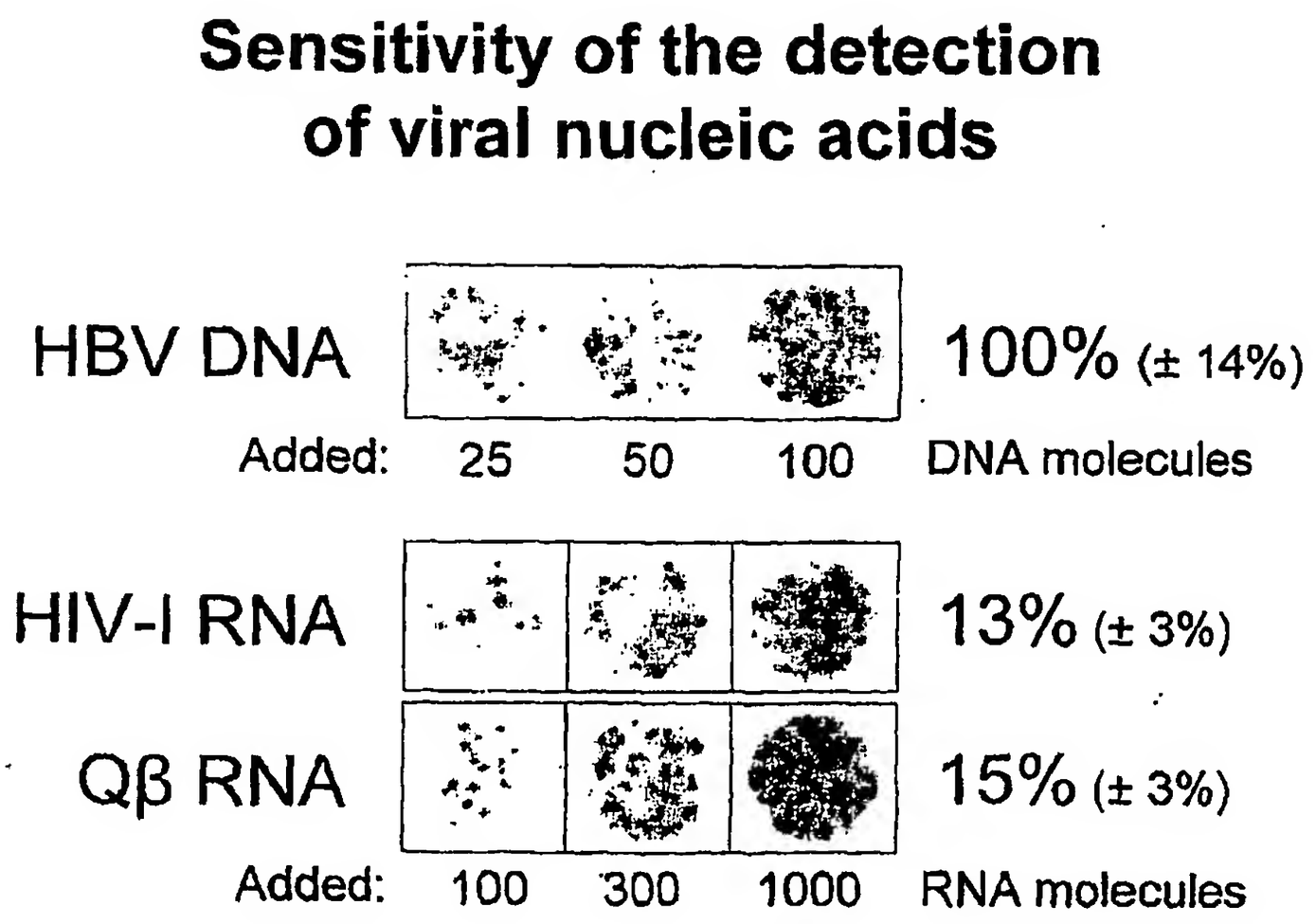

Sensitivity of the detection
of viral nucleic acids
HBV DNA
100% (± 14%)
Added: 25 50 100 DNA molecules
HIV-I RNA
13% (± 3%)
Qβ RNA
15% (± 3%)
Added: 100 300 1000 RNA molecules

Figure 1

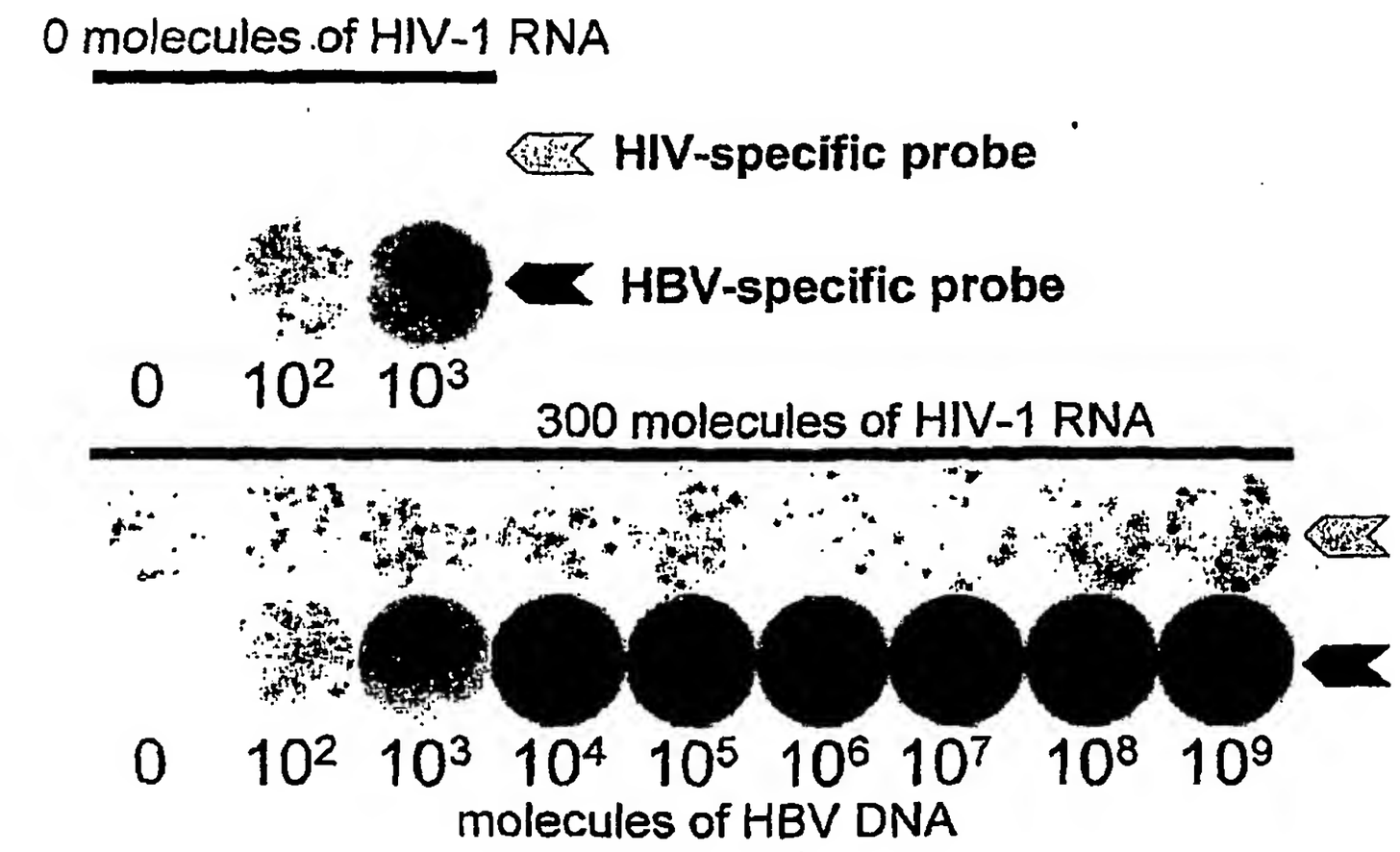

Detection of
HIV-1 RNA and HBV DNA in the same gel
0 molecules of HIV-1 RNA
HIV-specific probe
HBV-specific probe
0
10²
10³
300 molecules of HIV-1 RNA
0
10²
10³
10⁴
10⁵
10⁶
10⁷
10⁸
10⁹
molecules of HBV DNA

Figure 2

Solution PCR:
Background DNA synthesis interferes with target amplification

Target: 1000 HBV DNA molecules

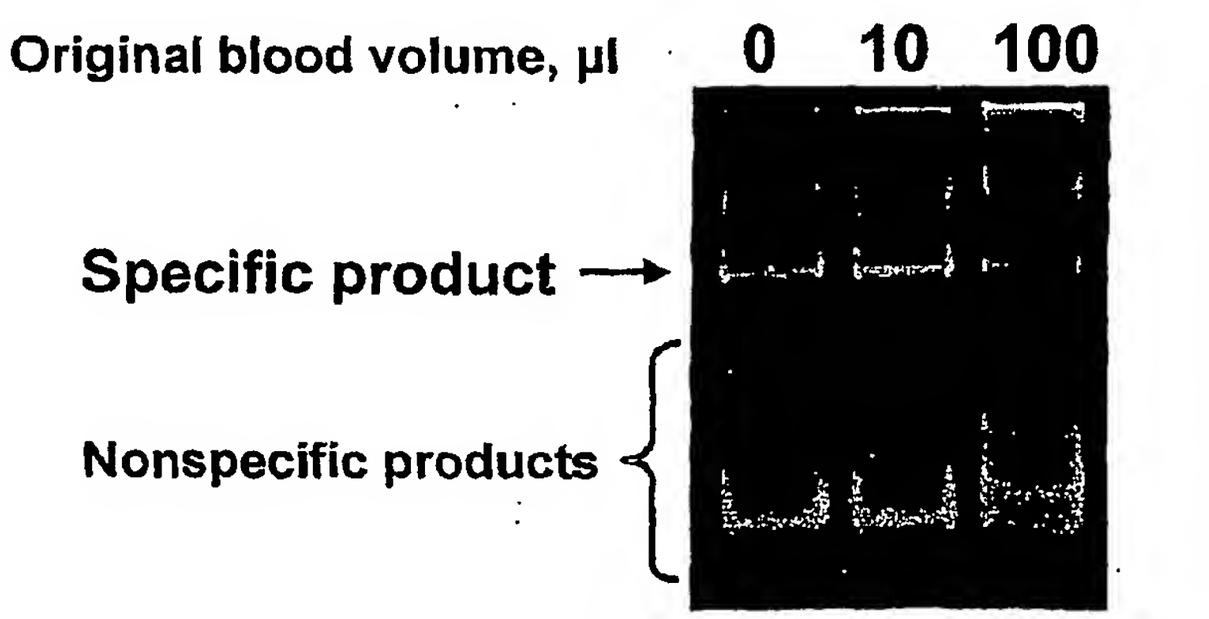
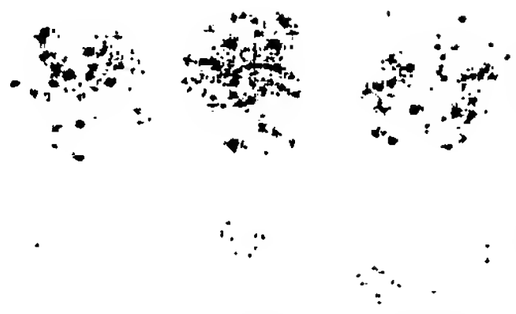


Figure 3a

Detection of HBV DNA in the presence of nucleic acids from human blood



				HBV DNA molecules
Original blood volume, µl	0	10	100	50
Human nucleic acids, µg	0	0.3	3	0
Genome equivalents	0	10^5	10^6	
Weight ratio to the target	0	10^{10}	10^{11}	

Figure 3b